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Short Communication

Functional properties of a 16 kDa protein translated from an alternative open reading frame of the core-encoding genomic region of hepatitis C virus

Arnab Basu,[1] Robert Steele,[2] Ranjit Ray[1,3] and Ratna B. Ray[1,2]

Departments of Internal Medicine[1], Pathology[2] and Molecular Microbiology and Immunology[3], Saint Louis University, St Louis, MO 63110, USA

Correspondence
Ranjit Ray
rayr@slu.edu

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Hepatitis C virus (HCV) often causes persistent infection in humans. This could be due in part to the effect of viral proteins on cellular gene expression. Earlier observations suggest that the HCV core protein expressed from genotype 1a modulates important cellular genes at the transcriptional level, affects programmed cell death (apoptosis) and promotes cell growth. Recently, different groups of investigators have reported the translation of an ~16 kDa protein (named F/ARFP/core+1 ORF) from an alternate open reading frame of the HCV core-encoding genomic region. The functional significance of this F protein is presently unknown. Thus, whether the F and core proteins have both shared and distinct functions was investigated here. The experimental observations suggested that the F protein does not significantly modulate c-*myc*, hTERT and p53 promoter activities, unlike the HCV core protein. Interestingly, the F protein repressed p21 expression. Further studies indicated that the F protein does not inhibit tumour necrosis factor alpha-mediated apoptosis of HepG2 cells or promote rat embryo fibroblast growth. Taken together, these results suggest that the F protein does not share major properties identified previously for the HCV core protein, other than regulating p21 expression.

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide, causing a spectrum of disease ranging from an asymptomatic carrier state to end-stage liver disease. The most important feature of persistent HCV infection is the development of chronic hepatitis in more than 50 % of infected individuals and the potential for disease progression to cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990; Alter, 1995; Kiyosawa & Tanaka, 2002; Koike *et al.*, 2000). Although limited replication of HCV occurs in some mammalian cells, successful purification of HCV has not been reported. It is therefore difficult to study the biological properties and pathogenicity of HCV. The virus genome contains a linear, positive-strand RNA molecule of ~9500 nt and encodes a single polyprotein precursor of ~3000 aa (Choo *et al.*, 1989). This polyprotein is cleaved by both host and viral proteases (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991), generating at least 10 individual proteins, the core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The core protein is located between aa 1 and 191 of the HCV polypeptide (Clarke, 1997).

The core protein may be the fundamental unit for encapsidation of genomic RNA to help in virus assembly. However, it has many intriguing properties. The core protein transcriptionally regulates a number of cellular promoters (Bergqvist & Rice, 2001; Jung *et al.*, 2001; Lai & Ware, 2000; Lee *et al.*, 2002; McLauchlan, 2000; Ray *et al.*, 1995a, 1997, 1998b; Srinivas *et al.*, 1996; Shrivastava *et al.*, 1998; Yoshida *et al.*, 2002). Since the core protein does not appear to bind DNA directly, the mechanism of transcriptional regulation remains to be elucidated. The HCV core protein protects against apoptosis mediated by c-*myc* in Chinese hamster ovarian cells and in tumour necrosis factor alpha (TNF-α)-sensitive human breast carcinoma (MCF7) and human hepatoma (HepG2) cells (Marusawa *et al.*, 1999; Ray *et al.*, 1998a; Shrivastava *et al.*, 1998). Functional involvement of the core protein in cell growth regulation was indicated from studies with primary human hepatocytes and rodent cells (Ray *et al.*, 1996; Chang *et al.*, 1998; Tsuchihara *et al.*, 1999). The HCV core protein leads to the development of hepatocellular carcinoma in transgenic mice (Moriya *et al.*, 1998), further suggesting that this protein may alter cellular physiology. These provocative results support the hypothesis that the core protein may participate in the pathogenesis of HCV-associated disease by modulating cellular gene expression and pathways of normal host functions.

Recently, different groups of investigators have reported the synthesis of proteins encoded from alternative open reading frames (ORFs) from the core genomic region (Xu *et al.*, 2001; Walewski *et al.*, 2001; Varaklioti *et al.*, 2002;

Vassilaki & Mavromara, 2003). In this study we investigated the functional role of the ~16 kDa protein (named F/ARFP/core+1 ORF) generated by a ribosomal frameshift between codons 8 and 11 of the HCV core-encoding genomic region (Xu *et al.*, 2001; Walewski *et al.*, 2001; Varaklioti *et al.*, 2002). The functional significance of this viral F protein remains unknown. It is a short-lived endoplasmic reticulum-associated protein (Roussel *et al.*, 2003; Xu *et al.*, 2003) and does not appear to play a major role in HCV replication because the subgenomic replicons, lacking the structural region of HCV, efficiently replicate in Huh-7 cells. This indicates that the structural proteins and, by consequence, the F protein are dispensable for HCV RNA replication (Gosert & Moradpour, 2002). The HCV core genomic region has the potential for generating smaller polypeptides by frameshifting or from an internal initiation codon (Choi *et al.*, 2003; Boulant *et al.*, 2003; Vassilaki & Mavromara, 2003). However, their expression in mammalian cells and functional relevance in HCV replication and pathogenesis remains to be elucidated. For simplicity, we will refer to the core as the protein product from nt 342 to 913 of genotype 1a (GenBank accession no. M62321).

Identification of the F protein raises the question as to which of the two proteins – the F or the core protein – encoded by the core genomic region from HCV genotype 1a is responsible for the previously observed functions (Ray & Ray, 2001). In this study, we examined the functions of the F protein compared with those from the HCV core genomic region. These results provided us with further insight into the functional properties of the F protein.

A partial cDNA clone (Blue4/C5p-1) of HCV genotype 1a containing the 5′-untranslated region, the core, E1, E2, p7 genes and a portion of the NS2 region (kindly provided by Michael Houghton, Chiron Corporation, Emeryville, CA, USA) was used as a template for amplification and cloning of the desired genomic regions. Different lengths of HCV core gene constructs, including portions of the structural regions of HCV, have been used by other investigators for understanding the processing and the functional role of the HCV core protein. Since we have used an HCV 1a core construct-encoding protein from aa 1 to 191 in most of our previous work, the same gene construct was made in this study with an N-terminal Flag tag for comparison of function with the F protein (Fig. 1a). The core genomic region was synthesized by PCR from Blue4/C5p-1 using sense (5′-CGTAGACCGGGATCCTGAGCACGAA-3′, containing a *Bam*HI restriction site) and antisense (5′-GAAGCGGGTCTAGAGCAAGCAAGA-3′, containing an *Xba*I site) primers and cloned in-frame into the pcDNA3-Flag mammalian expression vector (Ghosh *et al.*, 2003). The F gene was amplified by PCR using sense (5′-GCACCGGATCCATGAGCACGAATCCTAAACCTCAAAAAAAAAACA-3′, containing a *Bam*HI site) and antisense (5′-CCTGTTCTAGAGTTCACGCCGTC-3′, containing an *Xba*I site) primers. The amplified DNA was cloned in-frame into pcDNA3-Flag. This gene construct contained 10 aa from the core sequence followed by the +1 reading frame.

For *in vivo* expression, the N-terminally Flag-tagged core gene was expressed transiently in HepG2 cells using a recombinant vaccinia virus T7 polymerase system. After 24 h transfection, the cells were harvested and lysed for Western blot analysis. An anti-Flag murine monoclonal antibody (Sigma) or rabbit antiserum to an HCV core fusion peptide (aa 1–59 fused to GST, kindly provided by Arvind Patel, University of Glasgow, UK) was used as the primary antibody. An anti-mouse or anti-rabbit immunoglobulin coupled to horseradish peroxidase was used as the secondary antibody for detection of the viral proteins by chemiluminescence (ECL; Amersham). Western blot analysis using an anti-Flag antibody displayed expression of both ~21 kDa (core) and ~16 kDa (F) protein bands from the two ORFs of the HCV core genomic region (Fig. 1b). We also made a core mutant called core (9mt), mutated at aa 9 (Ray *et al.*, 2002), and cloned this in-frame with the Flag tag into the pcDNA3 vector. For this construct, we mutated K→R at codon 9, which abolishes the synthesis of the F protein (Fig. 1b). We did not observe expression of a smaller peptide from this mutant Flag–core construct, possibly because the smaller-sized protein was not detectable on the gel because of its size or because the ORF does not share the same methionine as the core protein. Western blot analysis using a rabbit antiserum to the core protein displayed expression of the HCV core protein only as a 21 kDa band (Fig. 1c). Therefore, our results suggested that both the 16 kDa and 21 kDa polypeptides are synthesized from the HCV core genomic region when transfected into mammalian cells.

We have observed previously that the HCV core protein has transregulatory properties against cellular genes. To examine whether the transregulatory effect occurs via the core or the F protein, we investigated the functional role of the F protein in transcriptional modulation of the cellular promoters c-*myc*, hTERT, p53 and p21. HepG2 cells were co-transfected with the F gene and the cellular promoter fused with the appropriate reporter gene as described previously (Ray *et al.*, 1995a). Empty vector DNA was used as a negative control for comparison. The total amount of DNA was kept constant in the transfection mixture by the addition of empty vector. Cytomegalovirus β-galactosidase was used as an internal control in determining transfection efficiency. A comparable number (<20% variation) of blue transfected cells expressing β-galactosidase was observed, suggesting a similar level of transfection efficiency. CAT or luciferase assays were performed at 48 h post-transfection to analyse changes in cellular gene expression. When cells were co-transfected with a human c-*myc*–CAT reporter construct and different effector plasmid DNAs, the HCV core gene or core (9mt) mutant enhanced CAT activity approximately fivefold from the human c-*myc* promoter, compared with the vector control (Fig. 2a). This enhancement was observed

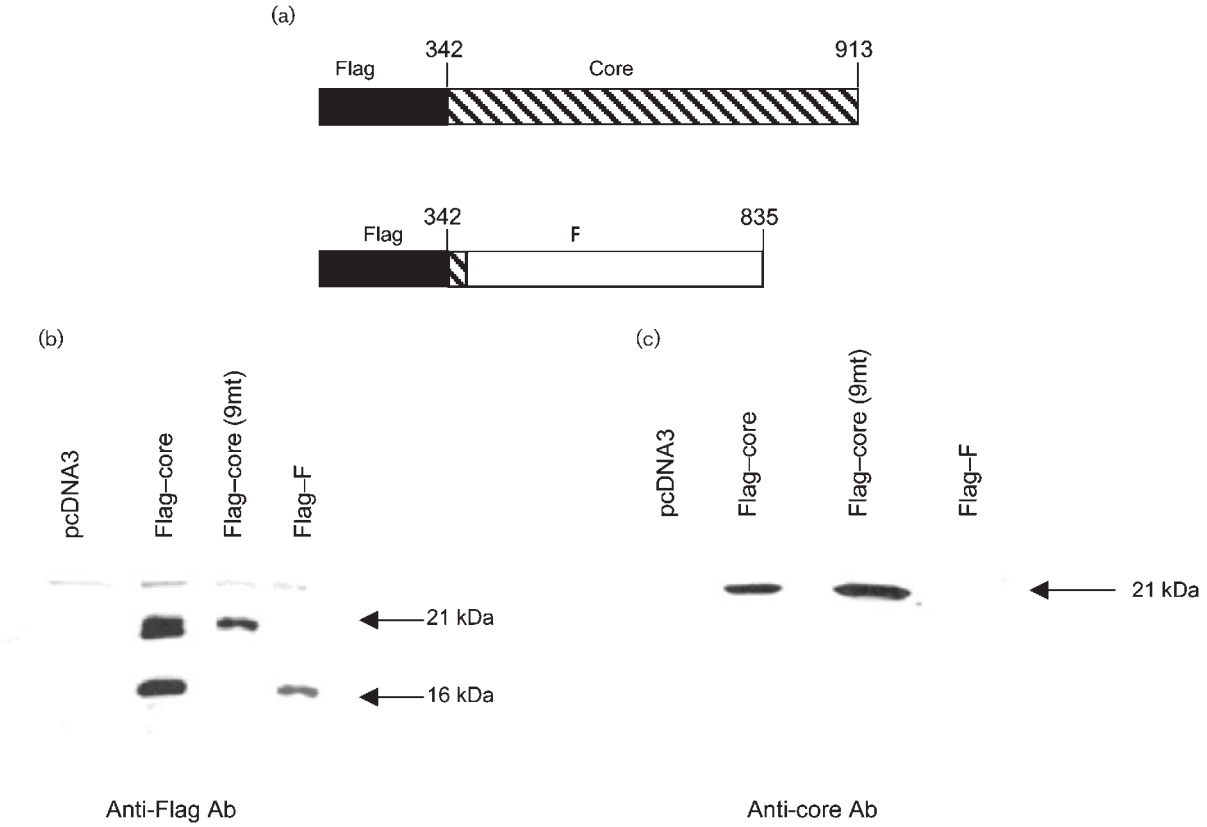

Fig. 1. Expression of the core and F proteins in HepG2 cells. (a) Schematic presentation of the Flag–core (top) and Flag–F (bottom) constructs. The core construct contains the coding regions of both the core and F protein. The +1 reading frame in the F protein is shown as an open box. The Flag tag, indicated by a black box, was fused to the 5′ end of the core or F coding sequence. The nucleotide positions of the HCV core protein from genotype 1a are shown. (b) Western blot analysis demonstrating F and core protein expression from the Flag–core and Flag–F plasmid constructs in HepG2 cells. Cells were transfected with Flag–core, Flag–core (9mt), Flag–F or the pcDNA3 vector (negative control). Proteins in cell lysates were separated by 15 % SDS-PAGE and Western blot analysis was performed with a specific monoclonal antibody to Flag. Arrows on the right indicate the positions of the core (~21 kDa) and F (~16 kDa) proteins. (c) Western blot analysis demonstrating core protein expression in HepG2 cells from the same Flag–core and Flag–core (9mt) gene constructs using rabbit antiserum to the core protein. The molecular masses of the protein bands were ascertained from the migration of standard protein molecular mass markers (Invitrogen).

to be dose-dependent (data not shown) as reported previously (Ray *et al.*, 1995a). On the other hand, transfection with the F gene displayed approximately twofold higher c-*myc* promoter activity and this modulation was not observed to occur in a dose-dependent manner over a range of 1–10 μg F plasmid DNA (data not shown). We have shown in a previous study that the N-terminal region of the core protein may play a role in c-*myc* promoter activation (Ray *et al.*, 1995a). The lack of c-*myc* activation by the F protein suggested that the N-terminal 10 aa, present in both the core and F proteins, are not involved in c-*myc* activation. Thus, the F protein did not appear to modulate c-*myc* promoter activity significantly, as was observed with the HCV core protein.

Since the core protein upregulates the hTERT promoter (Basu *et al.*, 2002), we examined whether the F protein plays a role in transcriptional modulation of hTERT. Cells were co-transfected with HCV F or core protein and an hTERT–luciferase reporter construct comprising ~800 bp of upstream sequence from the translation start site of hTERT gene. Analysis of luciferase activity in the cell lysates suggested that the F protein, unlike the HCV core or core 9mt, did not alter hTERT promoter activity (Fig. 2b).

We next analysed whether the F protein modulates p53 promoter activity, as has been shown for the HCV core protein (Ray *et al.*, 1997). HepG2 cells were co-transfected with a plasmid containing the human p53 promoter linked to the CAT reporter gene (p53–CAT) plus F plasmid DNA or empty vector DNA as a negative control. Core plasmid DNA was used in a similar co-transfection assay for comparison. Our results suggested that the F protein does not play a significant role in modulation of the p53

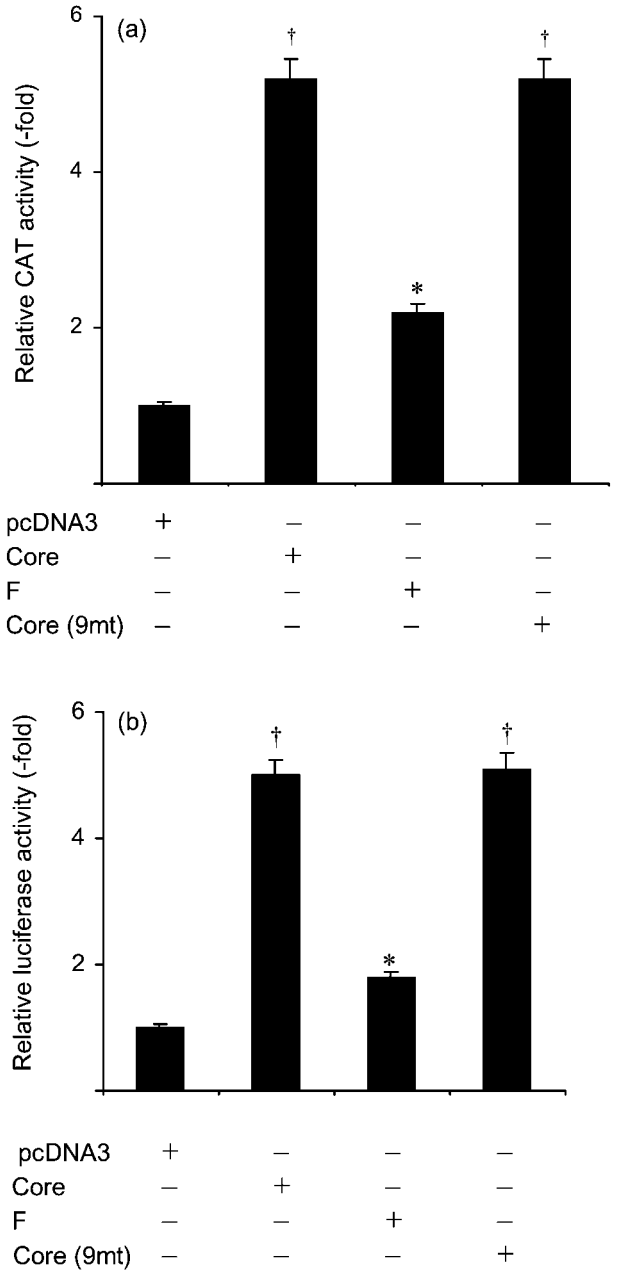

Fig. 2. Role of the F protein in transcriptional regulation. (a) Transcriptional regulation of the c-*myc* promoter by the HCV F protein in a transient transfection assay. HepG2 cells were co-transfected with 2 μg c-*myc*–CAT reporter gene and the core, core (9mt) or F plasmid DNA. Cell extracts were prepared 48 h post-transfection and analysed for CAT activity. Triplicate transfections were performed in each set of experiments and relative CAT activity is presented. The basal value was arbitrarily set at 1 and standard deviations are represented as error bars. †$P=0{\cdot}0006$ and *$P=0{\cdot}03$ relative to vector control. (b) Transcriptional regulation of the hTERT promoter by the HCV F protein in a transient transfection assay. An hTERT–luciferase reporter gene was co-transfected with core, core (9mt) or F plasmid DNA into HepG2 cells. Cell extracts were prepared 48 h post-transfection and analysed for luciferase activity. Triplicate transfections were performed in each set of experiments and relative luciferase activity is presented. The basal value was arbitrarily set at 1 and standard deviations are represented as error bars. †$P<0{\cdot}001$ and *P not significant relative to vector control.

promoter (Fig. 3a). In contrast, a reduction in p53 promoter activity was observed in the presence of the core gene. Thus, the results suggested that repression of p53 promoter activity was attributable to exogenous expression of the HCV core protein, but not the F protein.

We have shown previously that the HCV core genomic region represses p21 promoter activity in a p53-independent manner (Ray *et al.*, 1998b). To determine the effect of F protein expression, HepG2 cells were co-transfected with a human p21 promoter linked to the luciferase gene as the reporter construct together with core, core (9mt) or F plasmid DNA as the effector construct, using lipofectamine (Invitrogen). Introduction of the F gene into HepG2 cells significantly repressed p21 promoter activity (Fig. 3b). Core or core (9mt) protein expression also displayed repression of p21 promoter activity. The protein expression levels of p21 following core or F protein expression in HepG2 cells were also determined by Western blot analysis. Results suggested an approximately fourfold and 2·5-fold lower level of p21 in cells expressing core or F protein, respectively, compared with vector control cells (Fig. 3c). Repression of p21 transcription following HCV core gene expression from genotype 1b has also been observed by other investigators (Lee *et al.*, 2002; Nguyen *et al.*, 2003) and has been implicated as a mechanism of oncogene-mediated growth promotion. Thus, the transcriptional repression of the p21 promoter by the HCV F protein may promote cell growth.

TNF-α plays an important role in controlling viral infection. We and others have shown that the core protein protects against TNF-α-mediated apoptosis (Ray *et al.*, 1998a; Marusawa *et al.*, 1999). In this study, the role of the F protein on TNF-α-induced apoptosis was investigated. HepG2 cells were stably transfected with F plasmid DNA and cells were pooled to avoid artefacts arising from clonal analysis. We have shown previously that TNF-α treatment (20 ng ml^{-1}) of HepG2 cells induces apoptosis in the absence of actinomycin D (Ghosh *et al.*, 2000) and that the HCV core protein protects against TNF-α-mediated cell death (Ray *et al.*, 1998a). TNF-α treatment of empty vector- or F-transfected HepG2 cells for 24 h induced a fivefold increase in DNA fragmentation, as measured using a cell death ELISA kit (Roche), while cells expressing the HCV core protein inhibited DNA fragmentation (Fig. 4a). These results suggested that the HCV core protein protected cells from TNF-α-mediated apoptosis. However, HepG2 cells expressing the F protein displayed levels of cell death similar to the vector-transfected cells. Thus, the F protein did not appear to inhibit TNF-α-induced HepG2 cell death.

Early-passaged rat embryo fibroblast (REF 52) cells (Logan

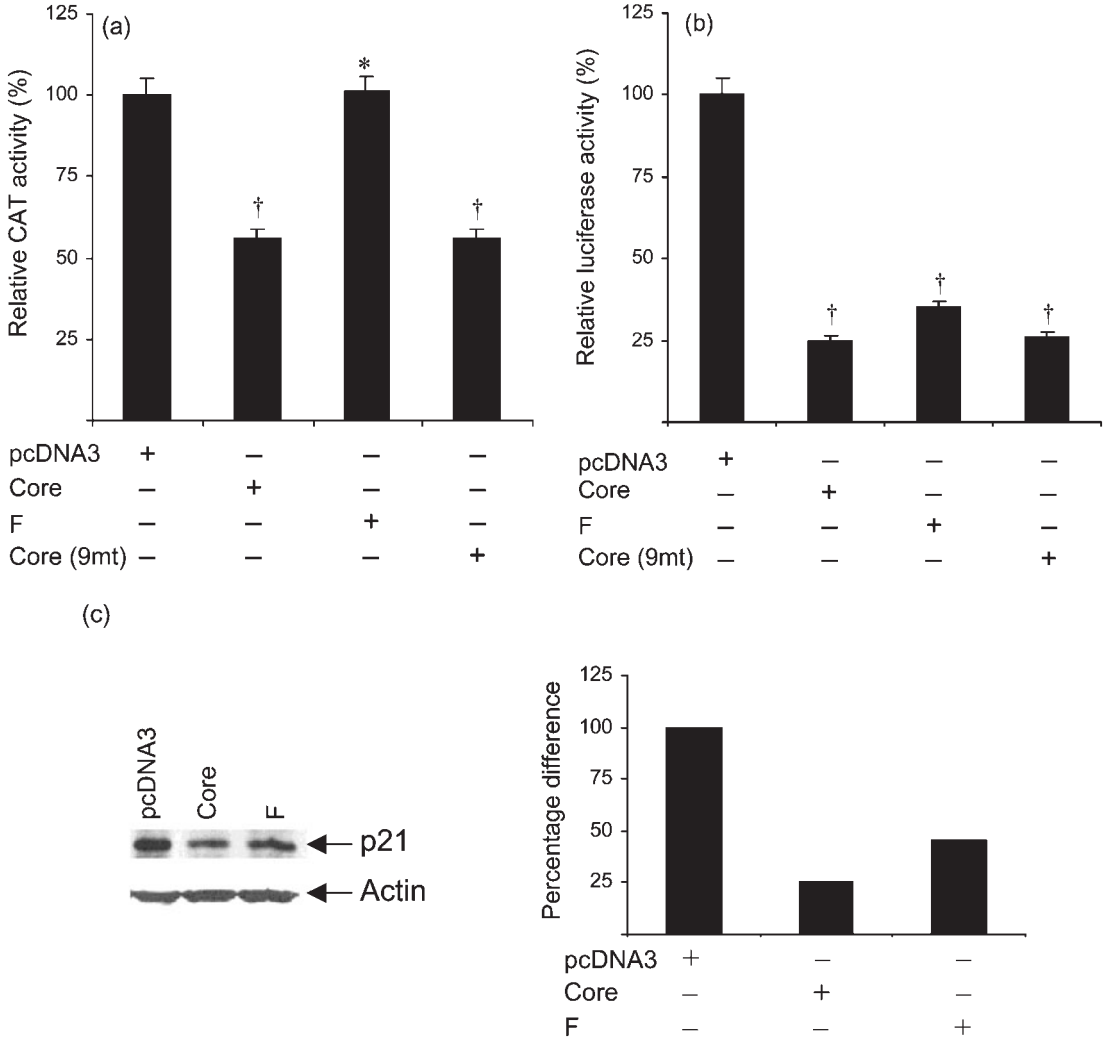

Fig. 3. (a) Regulation of p53 promoter activation by the HCV F protein. HepG2 cells were co-transfected with human p53–CAT reporter and HCV core, core (9mt) or F plasmid DNA (2 μg each). Cell extracts were prepared 48 h post-transfection and analysed for CAT activity. Triplicate transfections were performed in each set of experiments and relative CAT activity is presented. Basal value was arbitrarily set at 100 % and standard deviations are represented as error bars. †$P<0{\cdot}001$ and *P not significant relative to vector control. (b) Transcriptional regulation of the p21 promoter by HCV F protein in a transient transfection assay. A p21–luciferase reporter gene was co-transfected with HCV core, core (9mt) or F plasmid DNA (1 μg each) into HepG2 cells. Luciferase activity was measured 48 h post-transfection. Triplicate transfections were performed in each set of experiments and relative luciferase activity is presented. The basal value was arbitrarily set at 100 % and standard deviations are represented as error bars. †$P<0{\cdot}001$ relative to vector control. (c) Expression of p21 in HepG2 cells transfected with the empty vector DNA or the HCV core or F gene. Cell lysates were subjected to Western blot analysis using p21-specific antibody (left panel). The blot was re-probed with an antibody to actin for comparison of the amount of protein loaded in each lane. Arrows on the right indicate the respective proteins; their molecular masses were ascertained from the positions of a pre-stained molecular mass marker (Invitrogen). The relative abundance of p21 was estimated by densitometric scanning of the autoradiogram after normalization against actin and is shown as a histogram (right panel).

et al., 1981) were transfected with core, F or vector control plasmid DNA. Forty-eight hours post-transfection, cells were split at a 1 : 9 dilution, G418 (800 μg ml^{-1}) was added for neomycin gene selection and cells were selected for 3 weeks (Ray *et al.*, 1995b). Stable transfectants of REF 52 cells were pooled to avoid clonal artefacts and examined for growth. Transfected cells were morphologically indistinguishable. To determine the growth rate, an equal number of cells were plated and cell number was determined by trypan blue exclusion. Cell growth was also measured at 24 h intervals for 5 days using the CellTitre 96 aqueous non-radioactive cell proliferation assay (Promega). Cells were quantified from the formazan produced relative to blanks without cells. Relative cell numbers were

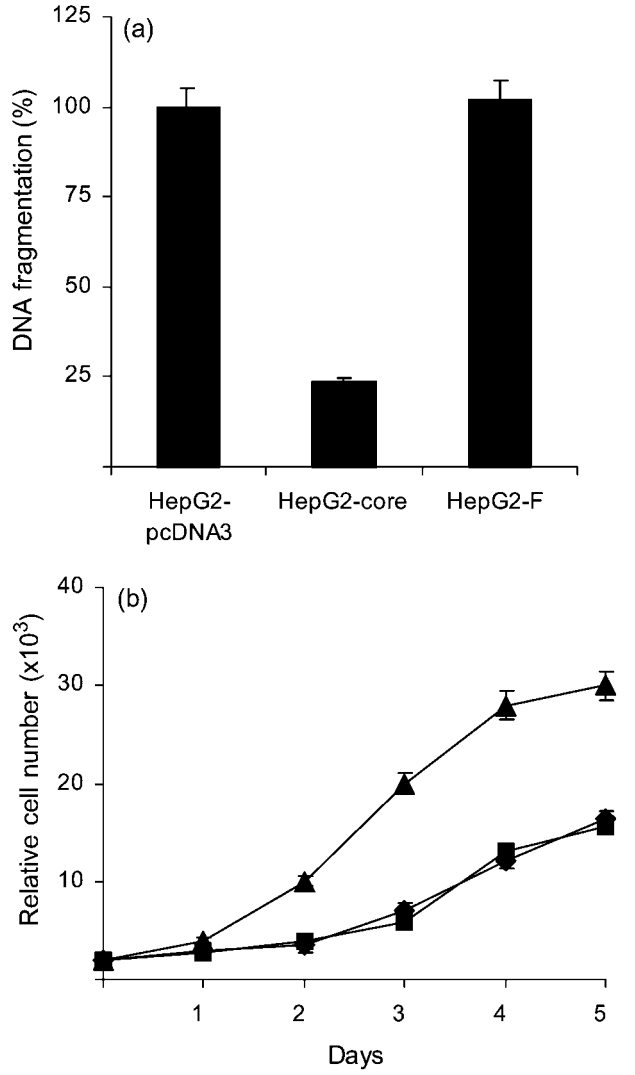

Fig. 4. (a) HCV F protein does not block TNF-α-mediated cytotoxicity. Quantification of apoptotic cell death in HepG2 cells transfected with pcDNA3 vector or the HCV core or F gene from cytosolic oligonucleosome-bound DNA by ELISA (Roche). Standard deviations from triplicate samples are presented as error bars. (b) Effect of F protein on REF 52 cell growth. REF 52 cells were transfected with plasmid DNA encoding the core or F genes or with the vector control. Stable transfectants were selected by treatment with G418 and cell viability/growth was assessed from triplicate culture wells at different time points using the CellTitre 96 aqueous non-radioactive cell proliferation kit (Promega). Results are presented as mean values from three different experiments ± SD. ◆, REF 52-pcDNA3; ■, REF 52-F; ▲, REF 52-core.

determined as described previously (Spanjaard *et al.*, 1997). Results from three independent experiments suggested that cells into which the F gene had been introduced had similar growth properties to vector-transfected cells, while core-transfected REF 52 cells displayed at least a twofold higher growth rate. Therefore, F protein expression did not appear to play a role in REF 52 cell growth modulation.

In conclusion, we have investigated the expression of the F protein *in vivo* and whether it shared some of the previously characterized activities of the HCV core protein. Similar observations were noted with and without the Flag tag in F or core gene constructs. Our results suggested that the F protein does not share the major functional properties identified previously for the HCV core protein and implicated in cell growth regulation. However, we do not rule out the possibility of functional activities of the protein(s) generated from other alternative reading frames of the HCV core genomic region; this will require further investigation. We previously observed transcriptional repression of p21 with the core genomic region from HCV genotype 1a (Ray *et al.*, 1998b). Recently, Yamanaka *et al.* (2002) reported that core protein expression in the nucleus decreased the amount of p21 by the p53-independent pathway. A different study (Nguyen *et al.*, 2003) also demonstrated that the mature form of the core protein inhibited p21 expression.

The F protein was not found in all HCV genomes available in GenBank. Translation of the F protein is easily detected by *in vitro* translation and transfection assays in some HCV isolates. Although seroconversion against the F protein in HCV-infected individuals has been reported (Walewski *et al.*, 2001; Varaklioti *et al.*, 2002), rabbit antiserum to a core fusion peptide (aa 1–59 fused to GST) did not recognize the F protein, implying that the first 10 aa of the core and F proteins do not constitute an antigenic site. Taken together, our results suggest that the F protein does not share the major functional roles of the core protein, except for modulation of the p21 gene. Therefore, the biological function of the F protein merits further investigation, especially with regard to its regulatory role on p21 promoter activity.

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